Identification and Characterization of Novel Microsomal Prostaglandin E Synthase-1 Inhibitors for Analgesia


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Running Title

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d. Abbreviations

COX-1 = cyclooxygenase-1; COX-2 = cyclooxygenase-2

mPGES-1 and mPGES-2 = Microsomal prostaglandin E synthase 1 and 2

cPGES = cytosolic prostaglandin E synthase; Monoiodoacetate (MIA)

Human whole blood (HWB)

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Abstract

Prostaglandin E$_2$ (PGE$_2$) plays a critical role in eliciting inflammation. Non-steroidal anti-inflammatory drugs and selective inhibitors of cyclooxygenase, which block PGE$_2$ production, have been used as key agents in treating inflammation and pain associated with arthritis and other conditions. However, these agents have significant side effects such as gastro-intestinal bleeding and myocardial infarction, since they also block the production of prostanoids that are critical for other normal physiological functions. Microsomal prostaglandin E$_2$ synthase (mPGES-1) is a membrane bound terminal enzyme in the prostanoid pathway, which acts downstream of cyclooxygenase 2 and is responsible for PGE$_2$ production during inflammation. Thus inhibition of this enzyme would be expected to block PGE$_2$ production without inhibiting other prostanoids and would provide analgesic efficacy without the side-effects. In this report, we describe novel mPGES-1 inhibitors that are potent in blocking PGE$_2$ production and are efficacious in a guinea pig monoiodoacetate model of arthralgia. These molecules may be useful in treating the signs and symptoms associated with arthritis.
Introduction

Prostaglandins play critical physiological roles in a variety of organ functions and serve as key mediators of inflammation, pain, and fever (Funk 2001, Smith 1989, Smyth et al 2009). PGE2, the most prominent prostanoid, is produced by sequential enzymatic reactions starting with the release of arachidonic acid from membrane glycerophospholipids by phospholipase A2, followed by conversion to endoperoxide PGH₂ by either cyclooxygenase-1 or cyclooxygenase-2 (COX-1 and COX-2, respectively), and finally the isomerization of PGH₂ to PGE₂ by terminal prostaglandin E₂ synthases (PGES). The intermediate PGH₂ also serves as a substrate for other synthases/isomerases leading to the production of TxA₂, PGI₂, PGD₂, and PGF₂α (Funk 2001). The COX-1 isoform is constitutively expressed and is responsible for the production of prostaglandins that preserve the gastric mucosa, while the COX-2 isoform is induced in response to cytokines in inflammatory conditions such as rheumatoid arthritis and osteoarthritis (FitzGerald 2009, Smyth et al 2009, Sujimoto and Narumiya, 2007). Nonsteroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective drugs provide symptomatic relief by blocking the production of PGE₂ through inhibition of both COX-1 and COX-2 or COX-2 alone (Rainsford 2007). However, NSAIDs are associated with gastrointestinal bleeding due to the inhibition of constitutively produced PGE₂, while COX-2 selective inhibitors have been associated with increased thrombotic risk and myocardial infarction, which may be due to the inhibition of other inducible prostanoids such as PGI₂, which is required for proper cardiovascular function (FitzGerald and Petrono 2001, Wang et al 2005, Mukerjee et al 2001, Fries and Grosser 2005, Grosser et al 2006). Thus, a selective inhibition of PGE₂ without adversely affecting other prostanoids would be expected to provide anti-inflammatory and analgesic effects without the negative side effects.

Microsomal prostaglandin E synthases 1 and 2 (mPGES-1 and mPGES-2) are terminal enzymes in converting PGH₂ to PGE₂. mPGES-1 is expressed at low levels and is up regulated in a variety of inflammatory conditions, while mPGES-2 is constitutively expressed in a variety of tissues (Jakobsson et al 1999, Samuelsson et al 2007, Tanikawa et al 2002). Inhibition of mPGES-1 either by gene deletion or
by pharmacological inhibition of activity demonstrates selective blockage of PGE$_2$ production and analgesic and anti-inflammatory activity (Trebino et al, 2003, Xu et al. 2008; Mbalaviele et al. 2010, Abdul-Malik et al, 2013; Bahia et al. 2014, Korotkova and Jakobsson 2014). These observations suggest that mPGES-1 plays a critical role in eliciting PGE$_2$ mediated inflammatory response and that blocking the enzyme activity is likely to provide analgesic and anti-inflammatory relief. Recently, we have identified and described novel mPGES-1 inhibitors that are highly selective, potent, and are orally available (Schiffler et al. 2015 a; Schiffler et al 2015 b). In this report, we show that these molecules are selective in blocking PGE$_2$ production, while exhibiting no inhibition of other prostanoids, such as PGI$_2$, at the concentrations tested. We also show that the compounds are effective in reducing pain in a guinea pig model of knee joint pain.
Materials and Methods

mPGES-1 inhibitors (Compound 1 and 2, reference mPGES-1 inhibitor MF-63).

The synthesis of MF-63 (Xu et al. 2008) and compounds 1 and 2 have been described (Schiffler et al. 2015 a; Schiffler et al 2015 b)

Human mPGES-1

Human mPGES-1 was purchased from Invitrogen (Cat# 97002RG, clone ID 6314722), was subcloned into pcDNA3.1, and was transiently expressed in HEK 293 cells. Microsomes were prepared from cell pellets based on published methods (Ouellet et al, 2002, Thoren 2003). In brief, cell pellets were sonicated in a buffer of 15 mM TRIS-HCl, pH 8.0, 0.25 mM sucrose, 0.1 mM EDTA, and 1 mM glutathione. The suspension was centrifuged at 5,000 x g for 10 min at 4°C. The supernatant fraction was loaded into Beckman Quickseal tubes (342413) and centrifuged at 185,000 x g for 90 min at 4°C using 70.1 Ti rotor. Pellets were resuspended in a buffer of 10 mM sodium phosphate, pH 7.0; 10 % glycerol, 2.5 mM glutathione; and Complete protease inhibitor cocktail (Roche). Final concentrations were 4.4 μg/mL microsomes and 1.69 μM PGH2. All dilutions were made using the above buffer. After a 2.5 minute incubation at room temperature, 2.5 μL/well SnCl2 in 0.5 N HCl was added to stop the reaction. PGE2 was quantitated by standard LC/MS analysis.

Guinea pig mPGES-1

Guinea pig mPGES-1 was cloned from IL-1 stimulated 104C1 (ATCC: CRL- 1405) by 5′-RACE and was subcloned into pQCXIN. HEK-293 cells were infected with the plasmid for 24 hours, and then expanded under selection in DMF/F12 3:1 (Invitrogen) + 10% FBS + 1 mg/mL G418. Cell pellets were processed into microsomes as described above. Activity was assessed as above with final concentrations of 15 μg/mL guinea pig mPGES-1 microsomes and 2 μM PGH2. PGE2 was measured by enzyme immunoassay (EIA, Cayman 500141) at a dilution of 1:1000.
Rat mPGES-1

Rat mPGES-1 cDNA was purchased from Open Biosystems (Cat# MRN1768-99238049, clone ID 7456259) and was subcloned into pQCXIN. HEK-293 cells were infected with the plasmid for 24 hours, and then expanded under selection in DME/F12 3:1 (Invitrogen) + 10%FBS + 1 mg/mL G418. Cell pellets were processed into microsomes as described above. Activity was assessed as above with final concentrations of 2 μg/mL rat microsomes and 2 μM PGH2 in 50 μL of buffer. PGE2 was measured by EIA (Cayman 500141) at a dilution of 1:1000.

Human mPGES-2

Human mPGES-2 was obtained from Open Biosystems (Cat# MHS101 L-14465, Clone ID 3946495) and was subcloned into pET21d base vector. It was expressed in BL21CDE3 cells. The enzyme was purified by nickel affinity and size exclusion chromatography. Activity was assessed in a buffer of 100 mM KPO4, pH 7.0, with 1 mM dithiothreitol. Final concentrations were 10 μg/mL human mPGES-2 and 2 μM PGH2. PGE2 was measured by EIA (Cayman 500141) at a dilution of 1:1000.

COX-1 and -2 Activity Assay

COX activity was assessed using a commercially available kit utilizing ovine COX-1 and human COX-2 (Cayman 560131).

Enzyme-Inhibitor Reversibility Studies by a Rapid Dilution Assay

A rapid dilution assay was performed as previously described in order to determine whether the inhibitor binding to the enzyme was reversible (Copeland, 2005). Briefly, human mPGES-1 was diluted into buffer at 100x its usual assay concentration. Compounds (or DMSO) were added to the enzyme at 10x their respective IC50s and incubated for 30 minutes at room temperature. PGH2 was diluted into buffer to give a 2 μM final concentration and a volume equal to 100x the enzyme + inhibitor volume was
added to initiate the reaction. At 20 second intervals, SnCl$_2$ was added to stop the reactions. PGE$_2$ was measured by EIA (Cayman 500141) at a dilution of 1:1000.

**A549 Epithelial Carcinoma Cell assay**

Human epithelial lung carcinoma cell line A549 was purchased from ATCC (CCL-185) and was maintained in Kaighn's F12 + 10% FBS, in 5% CO$_2$. For assay, cells were plated at 40,000/well in 96 well Falcon plates, 24 hours prior to treatment. Compounds were diluted in DMSO and were added at 1 μL/well, n=2, to give seven concentrations each. Cells were pretreated for 30 minutes at 37°C, 5%CO$_2$. rhIL-1β (R&D Systems) was added to give 0.2 ng/mL final. The treatment period was 18 hours. The conditioned medium was assayed for levels of PGE$_2$ PGD$_2$, TxB$_2$ and 6-keto PGF$_{1\alpha}$ by EIA (Cayman). The IC$_{50}$ values were calculated using Graphpad Prism nonlinear regression sigmoidal dose response curve fitting. Data are the mean ± sd of the indicated number of determinations.

**Human Whole Blood Assay**

Blood was collected from normal volunteer donors into sodium heparin vacutainer tubes (BD). Donors had not taken NSAIDs, aspirin, Celebrex, or glucocorticoids within two weeks of the donation. Blood was distributed into deep well polypropylene plates and compounds were added. The blood was pretreated at 37°C, 5% CO$_2$, in a humidified atmosphere, loosely covered, for 30 minutes, then LPS (Sigma, Serotype 0111:B4) was added to give a final concentration of 100 μg/mL. The plates were incubated for 20-24 hours, loosely covered, at 37°C, 5% CO$_2$, in a humidified atmosphere, on an orbital shaker at 100 rpm. The plates were sealed tightly with silicone cap mats and were chilled on ice for 1 hour, then centrifuged at 1800 x g, 10 minutes, 4°C, in an Eppendorf 5810R centrifuge. Plasma was removed from the cell layer and transferred to v-bottom polypropylene plates. One hundred microliters was quantitatively transferred to Costar cluster tube blocks and 400 μL/well methanol/internal standard was added. Solid phase extraction was performed using Waters HLB 30mg/bed 96 well plates and subjected to LC/MS/MS analysis. Calibration curves were obtained by plotting the peak area ratio PGE$_2$, ...
PGF\textsubscript{2α}, TxB\textsubscript{2}, and respective internal standard against the concentration. A weighted (l/concentration) least squares regression analysis was used to obtain a linear equation over the range of the calibration. The IC\textsubscript{50} values were calculated using Graphpad Prism nonlinear regression sigmoidal dose response (variable slope), with a fitted top of less than 1.5x the LPS control and a fitted bottom between zero and 1.5x the reference standard. Data are the geometric mean ± sd of determinations from six donors.

\textit{Intra-articular injection of LPS plus TNFα}

To assess the ability of mPGES-1 inhibitors to inhibit prostanoid production in the knee a guinea pig model was used. Male guinea pigs of around 300g were first dosed subcutaneously with either vehicle (95\% captex and 5\% NMP), 50 mg/kg MF-63 or 30 mg/kg diclofenac (an NSAID used as a positive control). One hour post dose animals were injected with either 50 \textmu L of saline into both right and left knees or with 100 \textmu g lipopolysaccharide (LPS-Sigma L2630 strain 0111:B4) plus 50 ng TNFα (R&D Systems 5053-TG-025/CF) in 50 \textmu L saline into both knees. Six hours post intra-articular injection, knee joints were lavaged to collect synovial fluid, and the fluid was measured for PGE\textsubscript{2}, PGI\textsubscript{2}, and PGF\textsubscript{2α} levels using EIA kits from Cayman.

\textit{Monoiodoacetate (MIA) pain model}

To assess pain efficacy, male Hartley guinea pigs (Charles River) of approximately 200-250 grams were used. To induce pain, the right knee of each guinea pig was injected with 0.3 mg MIA in 50 \textmu L of saline and the left knee with 50 \textmu L of saline. To test the efficacy of compounds, guinea pigs were either dosed 5 days (compound 1) or 9 days (compound 2) post MIA injection with vehicle (10\% Cremaphor EL in saline), 30 mg/kg of diclofenac (NSAID-positive control), two doses of compound 1 (50 or 75 mg/kg) or 2 doses of compound 2 (10 or 50 mg/kg). All dosing was subcutaneous at a dose volume of 5 mL/kg and the group size was n=6. Dose group was randomly assigned to each animal and dosing staggered by 10 minutes for each guinea pig. Pain was measured 4 hours post dosing via incapacitance testing. This test measures the difference in hind paw weight bearing between the MIA and
saline injected knees, and for the following studies each value represents the average of 3 separate measurements, each acquired over a 1 second period for each animal, with the values then averaged for each treatment group. Data are presented as means with standard error of the means (SEM). Data were evaluated by one way analysis of variance (ANOVA). Groups were compared to vehicle by Dunnett's test with a Bonferroni correction for comparison between groups. All statistical analyses were performed using JMP (version 8) statistical analysis program (SAS Institute Inc., NC). Differences were considered to be significant if the P value was less than 0.05.
Results

Preparation and Characterization of mPGES-1 Enzyme

In order to develop a reproducible mGES-1 activity assay, HEK293 cells were transiently transfected with human mPGES-1 cDNA and microsomal and cytosolic fractions were evaluated for mPGES-1 expression using immunoblot analysis. The results (Fig 1 A) demonstrate that the microsomal fraction contained mPGES-1. The untransfected cells contained no basal mPGES-1 protein. The microsomal fraction was used to determine enzyme activity.

The synthase activity was determined based on the ability of mPGES-1 (microsomal preparation diluted in phosphate buffer, pH 7) to convert PGH₂ (substrate) to PGE₂. A representative example of the effects of various substrate concentrations on PGE₂ production is shown in (Figure 1 B). The $K_m$ value (13.2 µM) of the enzyme activity is comparable to the value of 14 µM reported elsewhere (Ouellet et al 2002).

Identification of Novel and Selective mPGES-1 Inhibitors

The in vitro enzyme activity assay was utilized to identify and optimize novel chemical scaffolds as inhibitors. The structures of two optimized molecules (Compound 1 and Compound 2) representing two different scaffolds, MF-63 (a reference mPGES-1 inhibitor), and celecoxib are shown in Figure 2. Both compounds 1 and 2 (Figure 3 A and B) demonstrate full efficacy (100% inhibition) and concentration dependent inhibitory activity against mPGES-1 enzyme with IC₅₀ values of 0.241 ± 0.085 µM and 0.00094 ± 0.0059 µM, respectively. For comparison, the IC₅₀ value of the reference mPGES-1 inhibitor was 0.005 ± 0.003 µM (data not shown). Compounds 1 and 2 show very little activity against isolated mPGES-2, COX-1, or COX-2. Further, the COX-2 selective inhibitor (celecoxib) and non-selective NSAIDs (ibuprofen and diclofenac) show no activity against mPGES-1 at the concentration tested. To determine relevant animal species for evaluation as a disease model, the compounds were also tested against mPGES-1 from guinea pig and rat. The inhibitors showed potent activity versus guinea pig
mPGES-1 but very poor activity versus rat mPGES-1 at the concentration tested. A summary of enzyme activities of the mPGES-1 inhibitors as well as celecoxib are shown in Table 1. These results establish that the molecules are potent and selective.

We also assessed whether or not the compounds bound to the enzyme in a reversible fashion. A rapid dilution method was used to assess the nature of binding. mPGES-1 was incubated with concentrations of the inhibitors that were 10 fold higher than their respective IC$_{50}$ values. These solutions were then diluted 100 fold with the substrate solution resulting in inhibitor concentrations of 1/10 their IC$_{50}$ values. As shown in Figures 4A and 4B, the dilution resulted in a recovery of the enzyme activity over time. The results indicate that both compounds were reversible inhibitors.

**Selective Inhibition of mPGES-1 in IL-1$\beta$ Stimulated A549 Cells**

We next evaluated whether the mPGES-1 inhibitors were effective in blocking PGE$_2$ production in cells in response to an inflammatory stimuli. The human epithelial carcinoma cell line A549 produces a variety of prostanoids (PGE$_2$, PGI$_2$, PGF$_{2\alpha}$, and PGD$_2$) in response to IL-1 (Thoren et al 2000). Initially, we compared the effects of the reference mPGES-1 inhibitor (MF-63) and a COX-2 selective inhibitor (rofecoxib) on various prostanoids produced by IL-1 treated with A549 cells. A549 cells were pre-treated with various concentrations of compounds for 30 min, followed by IL-1$\beta$ treatment for an additional 18 h, and the conditioned media were analyzed for various prostanoids using EIA. The COX-2 selective inhibitor rofecoxib blocked the production of all prostanoids (PGE$_2$, PGI$_2$, PGF$_{2\alpha}$, and PGD$_2$) in a dose-dependent manner. The reference mPGES-1 inhibitor (MF-63) blocked PGE$_2$ production (Fig 5 A-E), but demonstrated varying levels of increase in other prostanoids suggesting shunting towards these molecules. We next evaluated effects of compounds 1 and 2 on IL-1$\beta$ treated A549 cells on PGE$_2$ and PGI$_2$ under similar conditions. The results (Fig 6 A, B, and C) demonstrate that mPGES1 inhibitors and celecoxib blocked PGE$_2$ production in a concentration dependent manner. Compounds 1 and 2 demonstrated IC$_{50}$ values of 0.87 ± 0.423 μM and 0.012 ± 0.006 μM, respectively. The mPGES-1
inhibitors also caused a 2-3 fold increase in PGI2 levels, demonstrating a shunting towards other prostanoids. In comparison, celecoxib, a COX-2 selective inhibitor blocked the production of both prostanoids. These results established that mPGES-1 inhibitors were effective in blocking IL-1β stimulated PGE2 production, and that the inhibitory effects were selective to PGE2 synthesis.

Selective Inhibition of PGE2 Production in LPS Stimulated Human Whole Blood in vitro

Previous studies with NSAIDs and coxibs have shown a correlation between the in vitro human whole blood IC80 and the plasma concentration achieved in vivo at clinically efficacious doses, thereby providing a basis for using biochemical potency to predict analgesic efficacy (Huntjens et al 2005). Therefore, we compared the activity of mPGES-1 inhibitors against the standard of care, celecoxib, in human whole blood in vitro. Compounds were added to freshly collected human blood obtained from normal volunteers who had not consumed any anti-inflammatory during the past 2 weeks. LPS (100 μg/mL) was added 30 min after the addition of the compounds, and after a 24 h incubation at 37°C, the prostanoids secreted into the plasma were quantified using LC/MS/MS. The results (Fig. 7) show that compound 1, compound 2, and celecoxib blocked PGE2 production in a concentration dependent manner. The IC50 values are 0.792 ± 0.267 μM; 0.015 ± 0.009 μM; and 0.551 ± 0.490 μM, respectively. Once again, clear differences were observed between mPGES-1 inhibitors and celecoxib on other prostanoids. While celecoxib blocked the production of both PGF2α and TxB2, mPGES-1 inhibitors showed no inhibitory activity against either of these prostanoids at the concentration tested. These results further demonstrate that the two mPGES-1 inhibitors selectively blocked PGE2 production in whole human blood cells with compound 2 being more potent than celecoxib.

mPGES-1 Inhibitors were Efficacious in Guinea Pig Monoiodoacetate Model of Pain

We next wanted to evaluate whether or not the mPGES-1 inhibitors were efficacious in an animal model of pain that is known to be at least partially mediated though PGE2 (Park et al 2014). Previous studies have suggested that a reference mPGES-1 inhibitor was effective in a guinea pig monoiodoacetate
model of pain (Xu et al. 2008). Since compounds 1 and 2 did not inhibit rat mPGES-1, but were effective against guinea pig mPGES-1 (Table 1), as a first step we evaluated whether the reference mPGES-1 inhibitor (MF-63) was effective in blocking PGE2 production in the knee joints of guinea pigs injected with TNFα + LPS. Preliminary studies established that the optimal inflammation was achieved by a combination of TNFα and LPS (data not shown). The guinea pigs were given either diclofenac, (30 mg/kg) or MF-63 (50 mg/kg) by subcutaneous injection 1 hr prior to intra-articular injection of LPS + TNFα. The joint fluid was collected by lavage with saline 6 h post TNFα+LPS injection and the lavage fluids were analyzed for PGE2, PGI2, and PGF2α levels. The results demonstrate that the LPS+TNFα stimulation of PGE2 was blocked in animals dosed with the reference mPGES-1 inhibitor or diclofenac (Fig 8). While diclofenac treated animals showed suppression of other prostanoids (PGI2 and PGF2α), the reference mPGES-1 inhibitor treatment inhibited only PGE2. These results demonstrate that the selectivity of mPGES-1 was also observed in vivo at least for the reference inhibitor.

We next evaluated the ability of mPGES-1 inhibitors to block the pain resulting from joint injury caused by the intra-articular injection of moniodoacetate. Previous studies have established that the injection of moniodoacetic acid (MIA) into the knee joint of rats and guinea pigs produces an acute inflammatory insult, joint degeneration, and pain (Schwartz et al, 1981, Williams and Thonar, 1989, Pomonis et al 2005, Malfait et al, 2013). The pain resulting from the joint injury can be measured via differential weight bearing of the hind legs using an incapacitance tester. In order to evaluate the analgesic efficacy, MIA-injected guinea pigs were dosed with vehicle, mPGES-1 inhibitors at the indicated doses, or 30 mg/kg of the NSAID diclofenac (vehicle saline). The pain was measured using incapacitance testing 4 hours post dosing. The mPGES-1 inhibitors (compounds 1 and 2) and diclofenac significantly inhibited pain versus vehicle, with the 75 mg/kg dose of compound 1 and 50mg/kg dose of compound 2 being significantly different from both the low doses of the respective compounds and diclofenac (p<0.05 Dunnett's test with Bonferroni correction for comparison between groups; Figure 9). These results establish that the mPGES-1 inhibitors were effective in a guinea pig model of pain.
Discussion

mPGES-1 is a terminal enzyme induced during inflammation, is responsible for the production of PGE2 and is a potential target for effective analgesic and anti-inflammatory activity without causing side effects (Trebino et al., 2003, Xu et al. 2008; Mbalaviele et al. 2010, Abdul-Malik et al., 2013; Bahia et al. 2014, Korotkova and Jakobsson 2014). In this paper we describe and characterize novel mPGES-1 inhibitors that are potent, selective, and are effective in a guinea pig model of pain. The two molecules exemplified in this paper are potent against human, dog, and guinea pig mPGES-1 enzymes and bind to the human enzyme in a reversible manner. They are highly selective and show no discernible activity versus mPGES-2, COX-1, and COX-2 enzymes. Both molecules are effective in blocking PGE2 production in IL-1 stimulated A549 cells, as well as in LPS stimulated human whole blood. Finally, they demonstrate efficacy in a guinea pig monoiodoacetate (MIA) model of pain.

NSAIDs and COX-2 inhibitors have been extensively used to treat the inflammation and pain associated with rheumatoid arthritis and osteoarthritis but show significant side-effects. Specifically, the cardiovascular side effects have been suggested to be due to a general blockage of all prostanoids (FitzGerald and Petrono 2001; Wang et al. 2005; Mukerjhee et al. 2001; Fries and Grosser 2005; Grosser et al. 2006). Thromboxane A2, a COX-1 mediated product produced in platelets, is critical in vasoconstriction and platelet aggregation. Conversely, COX-2 derived PGI2, produced in vascular smooth muscle cells and endothelial cells, is a vasodilator and inhibits platelet activation. Coxibs modulate the prothrombotic TxA2 production only marginally, decrease the production of antithrombotic PGI2, and create an alteration in the TxA2/PGI2 ratio that favors the prothrombotic status (FitzGerald and Petrono 2001, Wang et al. 2005, Mukerjhee et al. 2001, Fries and Grosser 2005, Grosser et al. 2006). Because of this cardiovascular liability, some coxibs have been withdrawn from the market (FitzGerald 2003, FitzGerald and Patrono 2001). Thus a significant need exists in developing safer alternatives to coxibs and NSAIDs.
mPGES-1 is an inducible integral membrane protein and acts as the terminal enzyme downstream of COX enzymes in producing PGE₂ from the intermediate PGH₂. This enzyme is normally co-expressed with COX-2 at very low levels in most tissues, is induced by various inflammatory signals such as IL-1, and TNFα, and is upregulated in synovial tissue, cartilage, and chondrocytes of osteoarthritis and rheumatoid arthritis patients (Tanioka 2000; Stichtenoth 2001; Yamagata 2001; Kojima 2002; Lazarus, 2002, Claveau 2003; Kojima 2004; Li 2005). Two other enzymes, mPGES-2 and cPGES, also have been suggested to be involved in PGE₂ production. mPGES-2 is expressed constitutively in several tissues along with COX-1 and is believed to play a house-keeping function (Murakami 2003). cPGES is present in the cytoplasm but its function in PGE₂ production is poorly understood (Lovgren et al 2007). Therefore, we have focused on identifying mPGES-1 inhibitors that are highly potent, selective, and orally active in relevant pre-clinical models.

Traditionally, NSAIDs and COX-2 selective inhibitors have been identified using in vitro enzyme activity, selectivity assays, human whole blood assay, and a variety of animal models that measure either pharmacodynamic end points such as PGE₂ levels or behavioral response such as nociception or hyperalgesia. Because the mPGES-1 inhibitors identified here did not inhibit rat or mouse mPGES-1 enzymes (Table 1), we were unable to utilize traditional rodent animal models for efficacy studies. A meta analysis of marketed NSAIDs and coxibs suggested that clinically efficacious doses of a variety of these drugs effectively blocked PGE₂ production in LPS-stimulated human whole blood at their respective IC₈₀ concentrations (Huntjens et al. 2005). Based on this observation we initiated a biomarker-driven approach which utilized IC₅₀ and IC₈₀ values from a human whole blood assay to determine compound efficacy (Werner et al. 2002). Our goal was to first identify molecules exhibiting high intrinsic potency in blocking PGE₂ production in LPS-stimulated human whole blood. Further, a single oral dose of 200 mg of celecoxib in humans provides blood levels of drug that, at Cₘ₉₅, reach the in vitro human whole blood IC₈₀ value and exceed the IC₅₀ value for duration of 6-8 hours (Werner et al. 2002). Using these data, we sought compounds with high intrinsic potency that afforded exposure in rats approaching
their IC<sub>50</sub> values in the human whole blood assay and remained above the HWB IC<sub>50</sub> for 6 hrs or more after oral dosing (Schiffler et al. 2015 a; Schiffler et al. 2015 b).

The initial assessment of enzyme inhibitory activity was done by testing the ability of compounds to block mPGES-1 activity of microsomal preparation of HEK293 cells transfected with a human mPGES-1 cDNA. The immunoblot analysis demonstrated the purity of the preparation and also showed that the enzyme was present in the microsomal fraction with very little being present in the cytosolic fraction (Figure 1). The <i>K_m</i> value of the enzyme preparation (13.2 nM) is in the range of reported activity for similar preparation (Ouellet et al. 2002). The compounds bound to the enzyme in a reversible manner (Fig 4).

PGE<sub>2</sub> inhibition and selectivity was demonstrated in two cell-based assays: a) in an IL-1β treated human epithelial cell carcinoma cells (A549) and b) human whole blood treated with LPS. In both the assays, the mPGES-1 inhibitors demonstrate selective inhibition of PGE<sub>2</sub>. The A549 cell line is capable of synthesizing various prostanoids (PGE<sub>2</sub>, PGI<sub>2</sub>, and TxA<sub>2</sub>) in response to IL-1. While NSAIDS and COX-2 inhibitors blocked all prostanoids, mPGES-1 inhibitors inhibited only the PGE<sub>2</sub> production. Actives from A549 cells were evaluated in human whole blood stimulated with LPS.

Both compounds 1 and 2 demonstrate shunting towards PGI<sub>2</sub>. As shown before, shunting towards other prostanoids is a mechanistic consequence of selective mPGES-1 inhibition (Trebin et al. 2003). The biological consequence of shunting to PGI<sub>2</sub> is unknown. Inhibition of PGI<sub>2</sub> production or function is associated with adverse cardiovascular function (Flavahan 2001; Arehart et al, 2008). However, it is important to consider that PGI<sub>2</sub> shows paradoxical activities that include both cardiovascular protective function as well as pro-inflammatory activity in arthritic models and conditions (Stitham et al. 2011). Prostacyclin deficient mice are resistant to an inflammatory and arthritic challenge and prostacyclin inhibitors were efficacious in models of inflammation and arthritis ((Honda et al. 2006; Pulichino et al. 2006). In contrast, COX-2, but not mPGES-1 deletion in mice, affords protection against thrombosis and...
hypertension (Yu et al, 2012, Chen et al, 2013). So far, cardiovascular protection has yet not been demonstrated using mPGES-1 inhibitors in animal models because of the species selectivity issues associated with the compounds. Irrespective of the potential dual role of PGI₂, our results indicate that the mPGES-1 inhibitors are effective in reducing pain in guinea pig models to a level comparable to an NSAID, diclofenac at the tested doses, although we do not know whether there is any PGI₂ produced in this model.

Although we did not utilize an animal model for compound optimization, we wanted to ensure that the key molecules were able to block a behavioral response that is known to be at least partially mediated through PGE₂. Since our compounds did not inhibit rat mPGES-1 at the concentration tested, but was effective against the guinea pig enzyme, we developed a guinea pig monoiodoacetate model of pain. Initially we established that a reference mPGES-1 inhibitor MF-63 selectively inhibited PGE₂ production in guinea pig knee joints injected with LPS+TNFα. In contrast, diclofenac, a traditional NSAID, blocked all prostanoids, further demonstrating in vivo evidence for the mechanism of action for this class of compounds. The two mPGES-1 inhibitors (compound 1 and 2) were efficacious in the guinea pig MIA model of pain.

We do not know whether the therapeutic potential of mPGES-1 inhibitors as anti-inflammatory/analgesic drug along with its potential safety features (cardiovascular and GI) can be demonstrated in the clinic. Current options are restricted to NSAIDs and celecoxib which display serious side-effects. The availability of selective PGE₂ inhibitors that do not alter the other prostanoids will facilitate the evaluation of these molecules as safer alternatives to NSAIDs and Coxibs.
**Author Contribution**

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Figure Legends

Figure 1. Characterization of mPGES-1

Panel A. HEK293 cells were transfected with cDNA for human mPGES1; the microsmal and cytosolic fractions were separated as described in Materials and Methods. An aliquot was subjected to gel electrophoresis followed by immunoblot analysis with anti mPGES-1 antibody (Cayman). Lanes 1 = starting material-cell homogenate; Lane 2 = low speed supernatant fraction; lane 3 = cytosolic fraction; lane 4 = microsomal fraction.

Panel B. The microsomal fraction was assayed for mPGES-1 activity using various concentrations of substrate (PGH2) and the PGE2 was quantitated by LC/MS/MS analysis.

Figure 2. Chemical Structure of mPGES-1 Inhibitors

Compounds 1 and 2 are newly described mPGES-1 inhibitors. Celecoxib, Rofecoxib (Cox-2 inhibitors) and MF-63, a reference mPGES-1 inhibitor, were used in some experiments.

Figure 3. Concentration-dependent Inhibition of mPGES-1 Activity by mPGES-1 Inhibitors

Various concentrations of compound 1 or compound 2 were first mixed with mPGES-1 enzyme (microsmal preparation) followed the addition of substrate PGH2. After a 2.5 min incubation at room temperature, the reaction was stopped by the addition of SnCl2. The product PGE2 was measured by LC/MS/MS. Panel A = Compound 1; Panel B = Compound 2.

Figure 4. Reversible Inhibition of mPGES-1 Activity by the inhibitors

A 10 fold IC50 concentration of each inhibitor was incubated with mPGES-1 followed by a 100 fold dilution with substrate, leaving a 1/10 fold IC50 concentration of inhibitor. This resulted in a recovery of enzyme activity over time, indicating that these are reversible inhibitors. Panel A = Compound 1; Panel B = Compound 2.
Figure 5. Characterization of Prostanoid Production in A549 Cells

A549 cells were first treated for 30 min with various concentrations of either MF-63 (reference mPGES-1 inhibitor) or Rofecoxib (COX-2 inhibitor), followed by treatment with hIL-1β for 18 h at 37°C in a humidified atmosphere of 95% air+5% CO₂. The conditioned media were analyzed for PGE₂ (Panel A), PGI₂ (Panel B), TxB₂ (Panel C), PGF₂α (Panel D), and PGD₂ (Panel E) using respective EIA kits from Cayman. The results are expressed as % inhibition of respective prostanoids relative to IL-1β treated control levels. The negative inhibition indicates higher levels of production relative to IL-1β control values.

Figure 6. Selective Inhibition of PGE₂ Activity in A549 Cells by mPGES-1 Inhibitors

A549 cells were pre-treated for 30 min with various concentrations of either compound 1 (Panel A), compound 2 (Panel B), or celecoxib (Panel C) followed by treatment with hIL-1β for 18 h at 37°C in a humidified atmosphere of 95% air+5% CO₂. The conditioned media were analyzed for PGE₂ or PGI₂ using respective EIA kits from Cayman. The results are expressed as % inhibition of PGE₂ or PGI₂ relative to IL-1β treated control levels. The negative inhibition indicates higher levels of production relative to IL-1β control values.

Figure 7. Selective Inhibition of PGE₂ Activity in Human Whole Blood Cells

Freshly collected human blood obtained from normal volunteers, who had not consumed any anti-inflammatory medication for two weeks, was treated with various concentrations of compounds 1, 2, or celecoxib for 30 min, followed by LPS (100 μg/mL) for 24 hours. The plasma was separated from cells and the prostanoids in the plasma were assayed by LC/MS/MS after a solid phase extraction (refer to Materials and Methods).

Figure 8. PGE₂ inhibition after Intra-articular injection of LPS plus TNFα in guinea pigs
Male guinea pigs were first dosed subcutaneously with either vehicle (95% captex and 5% NMP), 50 mg/kg MF-63 or 30 mg/kg diclofenac. One hour post dosing, animals were injected with either 50 μL of saline into both right and left knees or with 100 μg LPS + 50 ng TNFα in 50 μL saline into both knees. The synovial fluid was collected from the knee joints by lavage 6 hours post intra-articular injection and PGE2, PGI2 and PGF2α levels were determined using EIA kits from Cayman.

Figure 9. Assessment of Pain Efficacy of mPGES-1 Inhibitors in Guinea Pig MIA Model

Male Hartley guinea pigs were injected with 0.3 mg MIA in 50 μL of saline (right knee) or saline (left knee), and were dosed subcutaneously with compounds 5-9 days post MIA injection. The compound doses were as follows: vehicle (10% Cremaphor EL in saline), compound 1 (50 or 75 mg/kg), compound 2 (10 or 50 mg/kg), or diclofenac (30 mg/kg). The dose volume was 5 mL/kg and the group size was n=6. Pain was measured 4 hours post dosing via an incapacitance testing, as described in Materials and Methods. Data were evaluated by one way analysis of variance (ANOVA) and are presented as means with standard error of the means (SEM). Groups were compared to vehicle by Dunnett’s test with a Bonferroni correction for comparison between groups. All statistical analyses were performed using JMP (version 8) statistical analysis program (SAS Institute Inc., NC). Differences were considered to be significant if the P value was less than 0.05 (*/*/*).
Table 1

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Compound-1</th>
<th>Compound-2</th>
<th>Celecoxib</th>
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<tr>
<td>Human mPGES-1</td>
<td>0.241 ± 0.085, n=6</td>
<td>0.000944 ± 0.0059, n=10</td>
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<td>Guinea Pig mPGES-1</td>
<td>0.511, n=1</td>
<td>0.0044 ± 0.0097, n=2</td>
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<td>Rat mPGES-1</td>
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<tr>
<td>Human mPGES-2</td>
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<td>1.33 ± 0.92, n=2</td>
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<tr>
<td>COX-1</td>
<td>9% Inhibition @ 100 μM</td>
<td>-1% Inhibition @ 10 μM</td>
<td>31% Inhibition @ 100 μM</td>
</tr>
<tr>
<td>COX-2</td>
<td>16% Inhibition @ 30 μM</td>
<td>-1% Inhibition @ 30 μM</td>
<td>78% Inhibition @ 30 μM</td>
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</table>
Figure 2

A. Compound 1

B. Compound 2

C. Celecoxib

D. Rofecoxib

E. MF-63
Figure 3

A. Compound 1 vs. Log M

B. Compound 2 vs. Log M
Figure 4

A.

- No Inhibitor
- Compound 1

B.

- No Inhibitor
- Compound 2
Figure 5

A. PGE\textsubscript{2}  

B. PGI\textsubscript{2}  

C. TxB\textsubscript{2}  

D. PGF\textsubscript{2}\textalpha{}  

E. PGD\textsubscript{2}
Figure 6

A. Compound 1

B. Compound 2

C. Celecoxib

% Inhibition vs. Log M Concentration for different compounds and inhibition types.
Figure 7

A. 

B. 

C. 

D. 

E. 

F.
Figure 8
Figure 9

A.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Difference in hind paw weight bearing (Saline-MIA)</th>
<th>Day 5 post MIA</th>
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<tr>
<td>Vehicle</td>
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<tr>
<td>Diclofenac 30.0 mg/kg</td>
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<tr>
<td>Compound 1 50.0 mg/kg</td>
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<tr>
<td>Compound 1 75.0 mg/kg</td>
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</tbody>
</table>

B.

| Treatment                  | Difference in hind paw weight bearing (Saline-MIA) | Mean day 9 post-MIA |
|----------------------------|---------------------------------------------------|                    |
| Vehicle                    | ![Bar graph](image)                               |                    |
| Diclofenac 30.0 mg/kg      | ![Bar graph](image)                               |                    |
| Compound 2 10.0 mg/kg      | ![Bar graph](image)                               |                    |
| Compound 2 50.0 mg/kg      | ![Bar graph](image)                               |                    |